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Quantitative analysis of growth and volatile fatty acid production by the anaerobic ruminal bacterium *Megasphaera elsdenii* T81

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Abstract *Megasphaera elsdenii* T81 grew on either DL-lactate or D-glucose at similar rates (0.85 h^{-1}) but displayed major differences in the fermentation of these substrates. Lactate was fermented at up to 210-mM concentration to yield acetic, propionic, butyric, and valeric acids. The bacterium was able to grow at much higher concentrations of D-glucose (500 mM), but never removed more than 80 mM of glucose from the medium, and nearly 60 % the glucose removed was sequestered as intracellular glycogen, with low yields of even-carbon acids (acetate, butyrate, caproate). In the presence of both substrates, glucose was not used until lactate was nearly exhausted, even by cells pregrown on glucose. Glucose-grown cultures maintained only low extracellular concentrations of acetate, and addition of exogenous acetate increased yields of butyrate, but not caproate. By contrast, exogenous acetate had little effect on lactate fermentation. At pH 6.6, growth rate was halved by exogenous addition of 60 mM propionate, 69 mM butyrate, 44 mM valerate, or 33 mM caproate; at pH 5.9, these values were reduced to 49, 49, 18, and 22 mM, respectively. The results are consistent with this species' role as an effective ruminal lactate consumer and suggest that this organism may be useful for industrial production of volatile fatty acids from lactate if product tolerance could be improved. The poor fermentation of glucose and sensitivity to caproate suggests that this strain is not practical for industrial caproate production.

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Introduction

Volatile fatty acids (VFA) are monocarboxylic acids containing one to six carbon atoms, of which acetic acid (C_2) is the most familiar. These acids, which have a variety of industrial uses, can be produced by synthetic chemistry or by microbial fermentation. The lower VFA (C_1–C_4) are major fermentation products of many anaerobic microbes. By contrast, the higher VFA (C_5 (valeric) and C_6 (caproic)) are generally produced in only minor amounts by fermentative routes. These acids (hereafter referred to in their carboxylate anion form, e.g., acetate) are of particular interest as potential precursors to hydrocarbon fuels either via catalytic chemistry (e.g., the MixAlco process (Holtzapple and Granda 2009) or the valerate platform (Lange et al. 2010)) or via electrochemistry (e.g., Kolbe reactions; Torii and Tanaka 2001).

Although the higher VFA are of most interest as fuel precursors, few microbial species are known to produce these compounds in substantial amounts. The specialist bacterium *Clostridium kluyveri* produces butyrate and caproate by condensation of acetate-derived acetyl-CoA units, using ethanol as an oxidant; this species can also produce valerate in small amounts from ethanol and propionate or from *n*-propanol and acetate (Kenealy and Waselefsky 1985). However, the only bacteria reported to produce substantial yields of valerate and caproate from redox-neutral substrates (sugars or lactate) are two anaerobic species from the rumen: *Megasphaera elsdenii* (Rogosa 1972) and the lactate and amino acid-fermenting (but nonsaccharolytic) *Eubacterium pyruvativorans* (Wallace et al. 2004). *M. elsdenii*, originally isolated and described by Gutierrez et al. (1959) as *Peptostreptococcus elsdenii* but later

reclassified (Rogosa 1972), has been reported to produce butyrate and caproate from glucose and several other sugars. Additionally, this species produces butyrate and acetate from D-lactate and propionate and valerate from L-lactate. Differential product formation from glucose vs lactate is due to a repression of lactate racemase and an NAD-independent lactate dehydrogenase during growth on glucose (Hino et al. 1994; Fig. 1).

Despite the capacity for rapid growth of *M. elsdenii* and its unique VFA product profile, there have been no systematic studies aimed at examining maximal production of VFA by this organism or at characterizing fundamental growth parameters (growth rates and yields) in batch culture. We describe here the effect of culture conditions on growth, substrate utilization, and product formation by *M. elsdenii* T81, derived from the original culture of Gutierrez et al. (1959).

Materials and methods

Cultures and media *M. elsdenii* strain T81 (derived from the same culture as ATCC 17753) was kindly provided by Rhonda Zeltwanger, USDA-ARS-NCAUR, Peoria, IL, USA. Cultures were routinely cultivated in LTY and GTY medium containing lactate or glucose, respectively, as energy sources. The basal medium contained (per liter) the following:

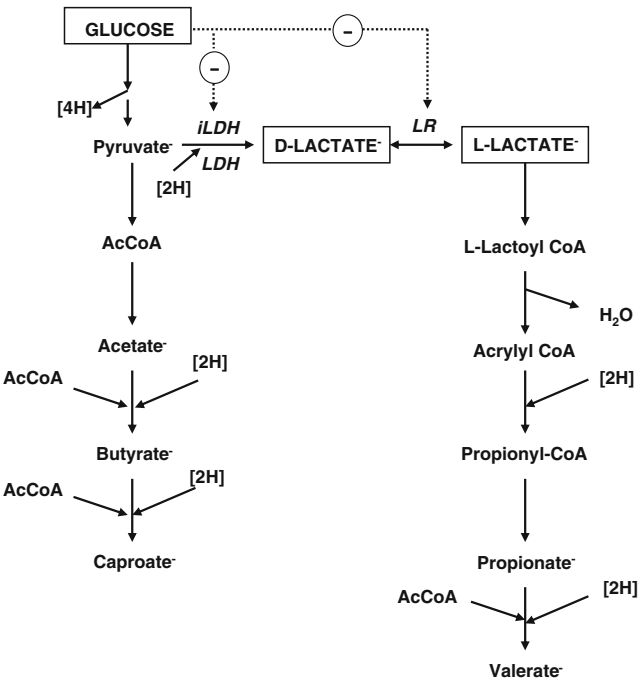

Fig. 1 Fermentation pathway of lactate and glucose by *M. elsdenii*. Glucose is known to repress synthesis of both lactate racemase (*LR*) and an NAD-independent lactate dehydrogenase (*iLDH*), but not the NAD-dependent lactate dehydrogenase (*LDH*). Adapted from Hino and Kuroda (1993)

1.25 g yeast extract (YE); 1.17 g each NaCl and $(NH_4)SO_4$; 0.065 g each $CaCl_2 \cdot 2H_2O$ and $MgSO_4 \cdot 7H_2O$; 0.026 g each $MnSO_4 \cdot H_2O$, $FeSO_4 \cdot 7H_2O$, and $ZnSO_4 \cdot 2H_2O$; and 0.0026 g $CoCl_2 \cdot 2H_2O$. Additionally, Trypticase was provided at 1 to 18 g/L, depending on the experiment. This basal medium (8.0 mL) was dispensed into Balch tubes under a CO_2 gas phase, sealed with butyl stoppers and aluminum crimp seals, and autoclaved prior to aseptic addition of sterile, N_2-gassed solutions of KH_2PO_4 (0.40 mL of 22.5 gL^{-1}), Na_2CO_3 (0.40 mL of 80 gL^{-1}), cysteine HCl (0.5 mL of 25 gL^{-1}), and energy source (0.20 mL of 4 M Na DL-lactate or 50 % (*w*/*v*) glucose).

Growth and metabolic products Growth was monitored turbidimetrically at 525 nm in a Spectronic 20 colorimeter (Bausch and Lomb). Maximum specific growth rate constants were determined by linear regression of ln (OD_{525}) vs time during exponential growth phase (typically over the range of ~2 to 6 h after inoculation). Soluble fermentation products were quantified by HPLC as described previously (Weimer et al. 1991), except that the pump module was a Varian ProStar and the autosampler was a Varian 410. Residual glucose was determined by the dinitrosalicylic acid colorimetric method of Miller et al. (1960). H_2 was determined by gas chromatography (Weimer 2010).

Fermentation balance experiments were conducted in triplicate volume-calibrated 160-mL serum vials that contained 100 mL of medium supplemented with 100-mM concentrations of energy source (DL-lactate or D-glucose) plus 1 g YE and 2.5 g Trypticase per liter. Vials were inoculated with 1 mL of an overnight culture grown on the same medium. After 48 h of incubation at 39 °C, the headspace was assayed for H_2, and two subsamples (40 mL) of the culture of each replicate were centrifuged (10,000 × *g*, 20 min). The supernatants were analyzed for soluble fermentation products and residual substrate. The pellets were resuspended in a minimal amount of saline (9 gL^{-1} NaCl), transferred to microcentrifuge tubes, and then centrifuged at 12,000 × *g* for 10 min. The pellets were resuspended in saline and centrifuged again, and the supernatant was discarded. The pellets were freeze-dried, resuspended to a 500-μL volume in 0.2 N NaOH, and heated in a boiling water bath for 20 min; the heated suspension containing cell lysate was cooled to room temperature and adjusted to a pH of approximately 5 with 192 μL of 10 % (*v*/*v*) glacial acetic acid. This suspension was analyzed for protein (Bradford 1976) using Coomassie Plus dye reagent (Thermo Scientific) with lysozyme as the protein standard. The same suspension was also analyzed for glycogen following hydrolysis with α-amylase/α-amyloglucosidase and subsequent glucose detection in supernatants (obtained by centrifugation as above) using glucose oxidase/peroxidase, as described by Hall (2011); the latter assay was modified to operate at 20-fold reduced volumetric scale.

Results

Growth on lactate M. elsdenii T81 grew with DL-lactate as energy source at concentrations up to 210 mM (Fig. 2a). At these concentrations, maximum specific growth rates were nearly identical (0.85 h^{-1}); nearly all of the added lactate was consumed (<0.3 mM residual), and the amounts of acetate and propionate produced (from D- and L-lactate, respectively; Hino and Kuroda 1993) were nearly equal. Likewise, butyrate and valerate were produced in nearly identical amounts (from D- and L-lactate, respectively; Hino and Kuroda 1993), at approximately half the concentrations of acetate and propionate. Only traces (<1 mM) of caproate were detected. Growth was completely inhibited at an initial lactate concentration of 260 mM. Fermentation balance calculations on 48-h batch cultures (Table 1) indicated nearly complete (>97 %) recovery of lactate carbon in measured products, including bacterial cells. Growth rate on DL-lactate was independent of initial culture pH (μ= 0.66 h^{-1}) within the pH range of 5.0 to 6.6 but declined substantially (μ=0.17 h^{-1}) at pH 4.65. Conversion of lactate to VFA typically increased culture pH by ~0.2 units, due to the higher pK_a of the VFA products relative to lactate (pK_a ~ 4.8 vs 3.8, respectively) and the decrease in total molar acid concentration due to longer chain VFA synthesis.

Growth on glucose Growth rate on glucose, calculated from optical density measurements, was similar to that on lactate (~0.85 h^{-1}). At increasing initial glucose concentrations, successively higher amounts of glucose remained in the culture medium, and the maximum amount of glucose consumed was only ~80 mM, although culture pH typically did not decrease below 5.5. Glucose fermentation (Fig. 2b) yielded butyrate and caproate as the major fermentation products, and neither propionate nor valerate was produced (Table 1). Unlike growth on lactate, growth on glucose was accompanied by a small amount of H_2S production, sufficient to turn the medium to light gray but without a distinct black ferrous sulfide precipitate. Fermentation balance calculations from 48-h batch cultures (Table 1) indicated a poor recovery of substrate carbon in products calculated on the basis of substrate consumed, suggesting that much of the glucose removed from the medium was not fermented. Analysis of cell pellets revealed extensive accumulation of intracellular glycogen (30.2 mg glycogen (mg cell protein)$^{-1}$). Nearly 60 % of the glucose removed from the medium was accounted for in glycogen, in addition to the 35 %

Table 1 Fermentation product balances for *M. elsdenii* T81 grown on DL-lactate or D-glucose[a]

	mol product/100 mol substrate consumed from	
	DL-Lactate[b]	D-Glucose[c]
Product		
Acetic acid (Ace)	27.6±0.3	4.8±0.4
Propionic acid (Pro)	29.5±0.7	0
Butyric acid (But)	9.8±0.4	13.3±1.0
Valeric acid (Val)	8.0±0.1	0.2±0.1
Caproic acid (Cap)	0.1±0.04	10.9±0.3
H_2	27.2±0.9	27.5±1.6
CO_2 (calculated)[d]	55.2±0.8	63.7±3.8
Cells[e]	2.5±0.3	4.6±0.4
Glycogen accumulated (% of substrate mass removed)	0.7±0.1	58.7±8.8
C recovery (%)[f]	98.2±1.3	95.0±8.9
O/R index[g]	1.22±0.02	1.22±0.03

[a] Results are mean values of triplicate vials ± S.E.M.

[b] Based on consumption of 97.6 mM Na–DL-lactate

[c] Based on consumption of 40.7 mM D-glucose

[d] Calculated as Ace+2(But)+Val+3(Cap); see Fig. 1

[e] Estimated from protein content of cell pellets, assuming that cells are 50 % protein and that the organic fraction of cell mass has an empirical formula of $C_5H_7O_2N$ (Pavlostathis et al. 1988)

[f] Includes accumulated glycogen

[g] Oxidation/reduction index calculated as described by Wood (1961), but including cell mass

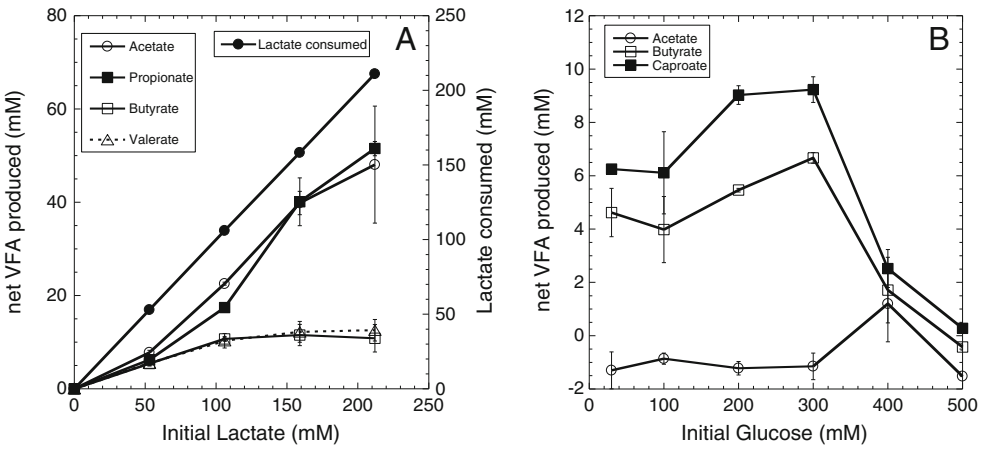

Fig. 2 Effect of substrate concentration on product formation from Na–DL-lactate (**a**) or glucose (**b**) by *M. elsdenii* T81. Results are mean values of duplicate tubes. *Error bars* indicate 1 standard deviation from the mean

accounted for in fermentation end products and nonglycogen cell mass. By comparison, glycogen was 50-fold lower (0.6 mg glycogen (mg cell protein)$^{-1}$) in cells grown 48 h on DL-lactate, and glycogen accounted for only 0.7 % of the lactate removed from the cultures.

Cultures simultaneously presented with DL-lactate and glucose did not use glucose until almost all of the lactate had been consumed, regardless of whether inocula had been pregrown on glucose (Fig. 3) or on lactate (data not shown). Both acetate and propionate accumulated to similar levels (~20 mM) early in the fermentation but were later consumed during the formation of butyrate, valerate, and caproate.

Effect of acetate on lactate and glucose fermentations DL-Lactate fermentations conducted for 24 h produced acetate at concentrations approximately one-half that of the initial lactate concentration, apparently due to the conversion of the L-isomer (Hino and Kuroda 1993). Growth of cultures on DL-lactate in the presence of increasing concentrations (10–50 mM) of added acetate had relatively little effect on the concentrations of specific VFA products (Fig. 4a). By contrast, glucose fermentations conducted for 24 h contained only low concentrations (~2 mM) of acetate, similar to or below those in control cultures inoculated into medium lacking glucose or other fermentable substrates. Moreover, glucose fermentations conducted with increasing levels of added acetate (10–50 mM) displayed extracellular pool sizes of acetate that approximated the amount of acetate added, and produced nearly stoichiometric increases in butyrate (Fig. 4b), suggesting that production of butyrate was limited by the availability of acetate rather than by the availability of electron donor.

Effect of alternative substrates Growth rates and fermentation product yields on lactate or glucose were not altered by the presence of the potential electron donors formate, H_2, or glycerol or by the potential electron acceptors NO_3^- or SO_4^{2-}. Trypticase did not stimulate growth when added at concentrations greater than 1 gL^{-1}.

Growth inhibition by VFA Growth of *M. elsdenii* T81 was inhibited by VFA (Fig. 5) in a dose-dependent manner, with caproate and valerate more inhibitory than propionate and butyrate. Inhibition was stronger at lower pH. At an initial culture pH of 6.6, concentrations of exogenously added individual VFA required to reduce the maximum specific growth rate by one-half were approximately 60, 69, 44, and 33 mM for propionate, butyrate, valerate, and caproate, respectively. At an initial culture pH of 5.9, these concentrations decreased to 49, 49, 18, and 12 mM, respectively. By contrast, cells were remarkably resistant to acetate: At pH 5.9, growth rate on lactate was only reduced by 8 % upon inclusion of 240 mM acetate in the medium.

Discussion

Although strains of *M. elsdenii* are regarded to have little physiological and genetic diversity (Piknová et al. 2006), this species is unusual among bacteria in its ability to ferment both glucose and lactate under anaerobic conditions (Gutierrez et al. 1959; Marounek et al. 1989), a property it shares only with some strains of *Selenomonas ruminantium*. Because of its ability to rapidly ferment lactic acid, *M. elsdenii* has been used as a probiotic strain to minimize lactic acidosis in the rumen of cattle fed with diets high in grain (Meissner et al. 2010), whose starch component is rapidly fermented to lactate by *Streptococcus bovis* and other ruminal bacteria (Russell and Hino 1985). Despite its unique catabolism and rapid growth rate, the potential of *M. elsdenii* to produce industrially useful concentrations of VFA from any substrate has not been systematically examined.

This study presents the first complete fermentation balance for *M. elsdenii*, including VFA, fermentation gases, glycogen, and cell mass. These fermentation experiments reveal that strain T81 displayed many of the same fermentation characteristics of strain NIAH 1102 reported by Hino et al. (1991, 1994) or the four strains examined by Marounek et al. (1989). DL-Lactate

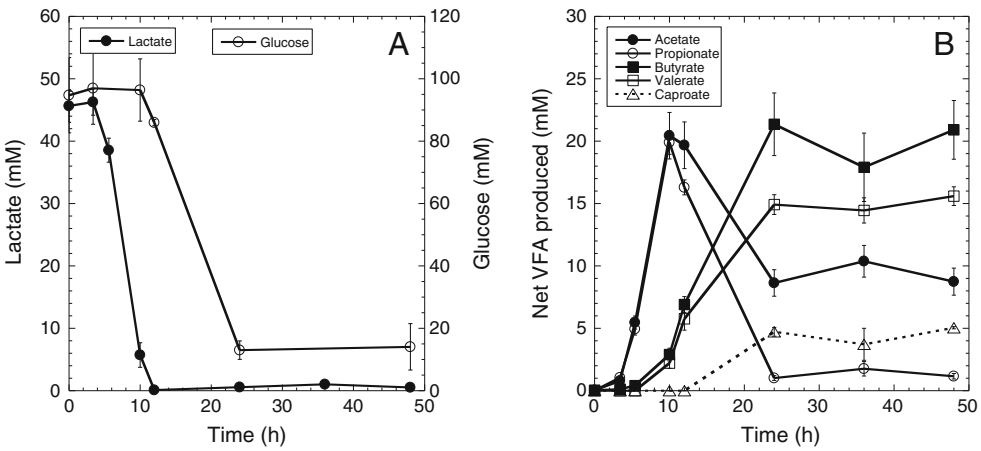

Fig. 3 Preferential utilization of DL-lactate over glucose by *M. elsdenii* T81 when presented with both glucose and lactate at time zero. Cells were pregrown on glucose. **a** Substrate disappearance. **b** Product formation. Results are mean values of triplicate tubes. *Error bars* indicate 1 standard deviation from the mean

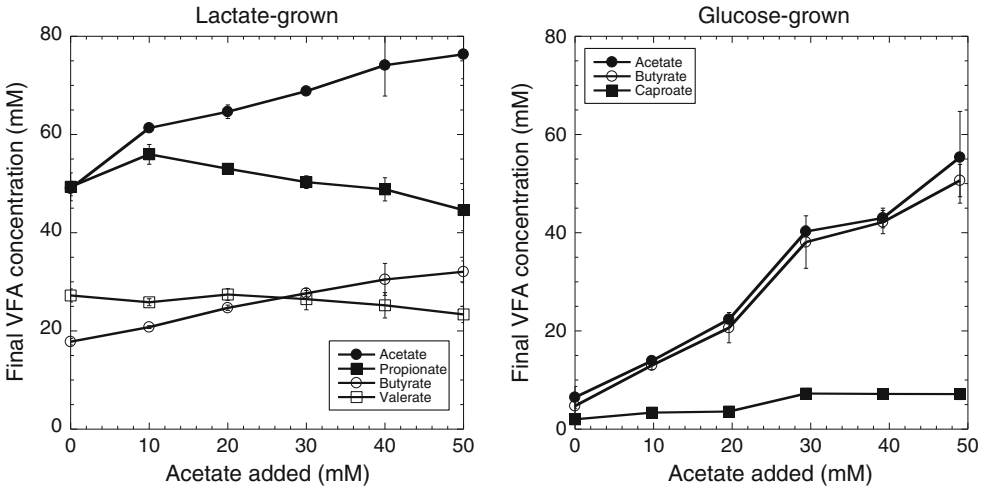

Fig. 4 Effect of different concentrations of exogenously added Na acetate (pH 6.5) on fermentation end product accumulation by *M. elsdenii* T81 grown on DL-lactate or glucose. Results are mean values of duplicate tubes. *Error bars* indicate 1 standard deviation from the mean

fermentation was accompanied by accumulation of propionate with smaller amounts of acetate, butyrate, and valerate, while glucose fermentation was accompanied by accumulation of butyrate with smaller amounts of acetate and caproate. Both valerate and caproate were produced only late in the fermentation and were also detected by Marounek et al. (1989) in 40-h incubations, but not by Hino and Kuroda (1993) and Hino et al. (1994) in 12-h incubations. Growth on lactate at initial concentrations of 210 mM or less resulted in nearly complete consumption of the substrate. However, no growth was observed on 260 mM lactate.

Although *M. elsdenii* T81 generally displayed similar growth rates on glucose and lactate, it preferred the latter substrate when both were presented together, and when fermenting glucose alone, it consumed a maximum of only 80 mM of the substrate, even when excess glucose was provided. Moreover, in cultures fed with glucose without either lactate or acetate, only one-third of the glucose removed from the culture was recovered in fermentation end products, with most of the remainder converted to glycogen. Poor product fermentation does not appear to be due to end-product inhibition, as VFA concentrations were well below (and pH values well above) those that result in only modest reduction in growth. Instead, poor fermentation of glucose appears to be due primarily to acetate limitation. Although exogenous addition of acetate had no effect on growth rate, it dramatically enhanced production of butyrate, suggesting that acetate is an important electron sink for this bacterium. Improved production of both butyrate and caproate was also observed in bisubstrate cultures in which lactate fermentation yielded acetate in concentrations sufficient to drive butyrate and caproate synthesis from glucose. The results are in accord

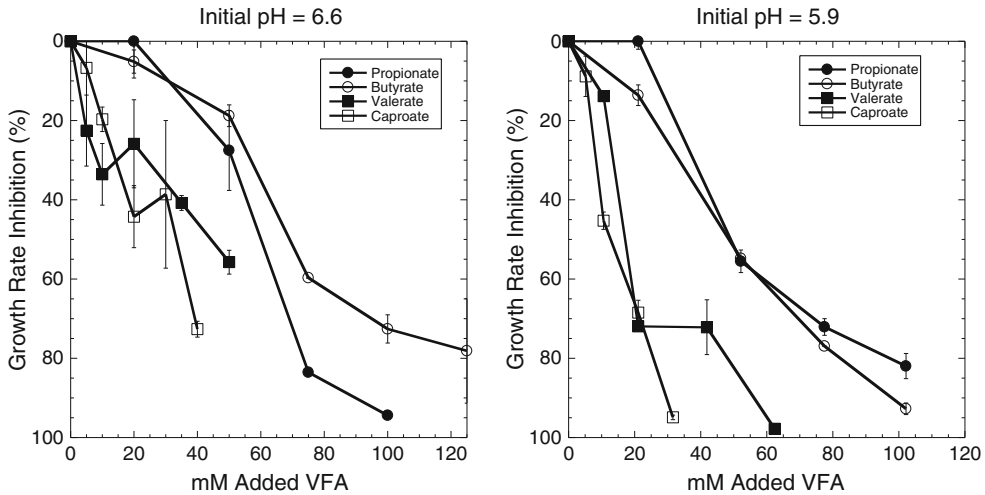

Fig. 5 Reduction of growth rate of *M. elsdenii* T81 by exogenously added individual VFA at initial culture pH values of 6.6 and 5.9. Inhibition of growth by propionate, butyrate, or valerate was determined using 80 mM DL-lactate as energy source (μ_{max}=0.53 h^{-1} at pH 6.6 and 0.64 h^{-1} at pH 5.9), while inhibition by caproate was determined using 55 mM glucose as energy source plus supplemental 10 mM Na acetate (μ_{max}=0.87 h^{-1}). VFA were added from 0.5 or 2.5 M stock solutions of Na VFA (pH 6.6 or 5.9) to achieve the indicated concentrations. Results are mean values of duplicate tubes. *Error bars* indicate 1 standard deviation from the mean

with those of Hino et al. (1991), who demonstrated the stimulatory effect of acetate on growth and VFA production from glucose in *M. elsdenii* NIAH 1102. Preferential utilization of lactate by this species underscores its important role as one of the few known lactate utilizers in the rumen (Meissner et al. 2010).

Growth yields, calculated from measured cell protein content, and cell composition assumed by Pavlostathis et al. (1988) were 0.024 and 0.087 g glycogen-free cells (g substrate utilized)$^{-1}$ for lactate and glucose, respectively. A higher growth yield on glucose is in accord with the observation of Hino et al. (1994) that 40 mM of DL-lactate was required to support growth to the extent obtained with 8 mM glucose. These low yields are probably not an artifact of cell lysis (which can occur in older cultures), as lysis would have resulted in loss of glycogen and thus a much lower carbon recovery than the 95–98 % that we observed (Table 1). Previously, Russell and Baldwin (1979) reported much higher yields of 0.24 to 0.41 g cells (g glucose removed)$^{-1}$ and a true catabolic growth yield (corrected for maintenance) of 0.54 g cells (g glucose removed)$^{-1}$ from continuous culture studies in which growth was measured by cell dry weight but without correction for glycogen content. The more modest growth yields of *M. elsdenii* observed in the present study should be advantageous from the standpoint of industrial fermentation of end products, as it permits higher yields of these products at the expense of cell growth.

Growth of *M. elsdenii* on either glucose or DL-lactate resulted in similar molar yields of H_2 (~0.25 molH_2 (mol substrate)$^{-1}$, Table 1). Diversion of reducing equivalents to H_2 production decreases available reducing equivalents for production of C_4–C_6 VFA from acetate and propionate and, in the case of lactate, H_2 production represent a particularly inefficient means of electron disposal (at pH 7, $E^{0\prime}$ of lactate/pyruvate= −190 mV and $E^{0\prime}$ of $H_2/2H^+$=−414 mV; Thauer et al. 1977). In *C. kluyveri*, an ethanol-oxidizing VFA producer with many physiological similarities to *M. elsdenii* including a low growth yield, H_2 production is stoichiometrically coupled to acetate synthesis and is necessary to provide substrate flux and entropic balance in the exergonic synthesis of VFA (Thauer et al. 1977). H_2 production probably has a similar function in *M. elsdenii*.

Although many bacteria can produce valerate from amino acid fermentation, few can produce this compound from other substrates. *C. kluyveri* has been reported to produce up to 8.5 mM valerate from *n*-propanol and acetate (Kenealy and Waselefsky 1985). The maximum amount of valerate accumulation reported in *M. elsdenii* cultures is 13 mM (Marounek et al. 1989), in which attempts to maximize accumulation were not made. By comparison, we have obtained concentrations of up to 25 mM (Fig. 2); we are unaware of higher concentrations of valerate for any species reported in the literature. Maximal VFA production by *M. elsdenii* is facilitated by a combination of high (but subtoxic) concentration of substrates and prolonged (2–3 days) incubation times. Industrial production of valerate would require substantial improvement in product concentration (probably in the 100-mM range) that could be gained in part by increasing tolerance to both substrate lactate and product valerate. By contrast, the poor fermentation of glucose by *M. elsdenii* typically results in accumulations of <10 mM caproate, well below the 110 mM readily achieved by *C. kluyveri* grown on ethanol and acetate (Weimer and Stevenson 2011); it thus appears that industrial fermentation of glucose by this strain is not practical.

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Conflict of interest Mention of specific products is for informational purposes only and does not constitute an endorsement or warranty over similar products that may also be suitable.

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